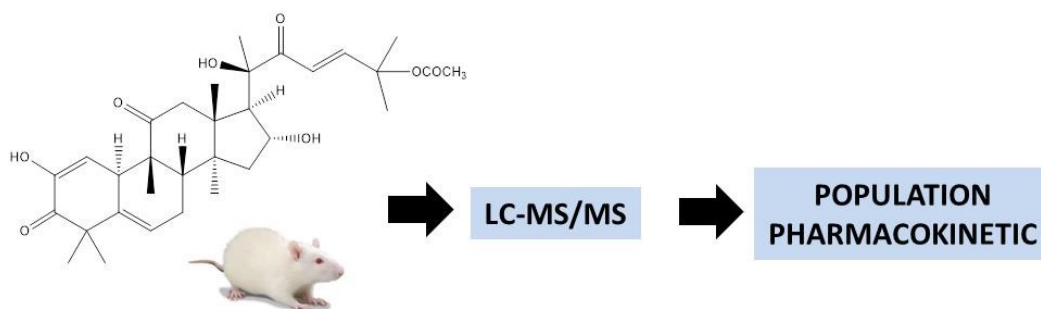# GRAPHICAL ABSTRACT

1 **Development and validation of a quantification method for cucurbitacins E and I**

2 **in rat plasma: Application to population pharmacokinetic studies**

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34   **ABSTRACT**

35   Cucurbitacin E is a potential drug candidate due to its anticancer activity, recognition of

36   its molecular targets, and synergism with other drugs used for cancer treatment.

37   However, the use of cucurbitacin E in clinical practice is not possible because of

38   important knowledge gaps in its preclinical and clinical pharmacokinetic characteristics.

39   Cucurbitacin E is hydrolyzed to cucurbitacin I in plasma and in human liver

40   microsomes. The aim of this study was to evaluate the population pharmacokinetics of

41   cucurbitacin E and of its metabolite cucurbitacin I in rats. The method for the sequential

42   analysis of cucurbitacins E and I in rat plasma was developed using LC-MS/MS.

43   Plasma aliquots of 50 µL were deproteinized with acetonitrile and clobazam was added

44   as internal standard. The extracts were injected into an RP-18 column and eluted with a

45   mobile phase consisting of a mixture of acetonitrile:water:methanol (32:35:33, v/v/v).

46   The method was precise and accurate, showing linearity in the range of 1-100 ng

47   cucurbitacin E/mL plasma and of 0.4-200 ng cucurbitacin I/mL plasma. The method

48   was applied to the pharmacokinetic evaluation of cucurbitacin E administered

49   intravenously to male Wistar rats (1 mg/kg). Serial blood samples were collected up to

50   24 h after administration. The plasma concentrations of cucurbitacin E were quantified

51   up to 16 h, while the plasma concentrations of cucurbitacin I remained below the limit

52   of quantification. A population pharmacokinetic model was developed for cucurbitacin

53   E using the NONMEM program, with adequate goodness of fit and predictive

54   performance. The following pharmacokinetic parameters were obtained: release time of

55   0.45 h, volume of distribution of 27.22 L, clearance of 4.13 L/h, and elimination half-

56   life of 4.57 h.

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58   **Keywords:** cucurbitacin E, cucurbitacin I, rats, LC-MS/MS, population

59   pharmacokinetics

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1. INTRODUCTION

Cucurbitacins found mainly in the family Cucurbitaceae are oxygenated tetracyclic triterpenes [1]. These compounds have a great pharmacological potential because of their broad spectrum of biological activities such as antimicrobial [2], anti-inflammatory [2,3], anti-HIV [4], antioxidant [5], cytotoxic and antitumor properties [6,7].

The current interest in cucurbitacins focuses on their antiproliferative and cytotoxic potential against a large number of human cancer cell lines, including colon, breast, liver, skin, lung, central nervous system, prostate, and nasopharyngeal cancer [6-11]. In this respect, promising targets of cucurbitacins in cancer therapy are being identified, including inhibition of the JAK2/STAT3 signaling pathway [12-14], rupture of the cytoskeletal actin and vimentin networks [12-13].

Although neglected for decades, cucurbitacin E (Figure 1) has gained special attention of many research groups due to its promising anticancer activity [8, 10, 12-13]. Its ability to disrupt the cytoskeleton and to inhibit the JAK2-STAT3 pathway, among other mechanisms, and the synergisms with other drugs used in cancer therapy have rendered cucurbitacin E a candidate for clinical evaluation [6, 9,15].

Preliminary studies on the metabolism of cucurbitacins have shown that cucurbitacin E is hydrolyzed to cucurbitacin I (Figure 1) in human plasma by paraoxonase [16] and in human liver microsomes by carboxylesterases [17]. The analytical methods for cucurbitacins described in the literature generally use plant extracts [11,16,18], buffer solutions and organic solvents [16,19] and HPLC with ultraviolet detection. The analysis of cucurbitacins in biological fluids is only described for cucurbitacin B in rat plasma by UPLC-MS/MS [20] and for cucurbitacin I in rat plasma using LC-MS [21]. No data are available regarding the development and validation of analytical methods for cucurbitacin E in biological fluids.

The present study reports for the first time the development and validation of a sequential analytical method for cucurbitacin E and its metabolite cucurbitacin I in rat plasma using LC-MS/MS. The analytical method was applied to pharmacokinetic studies of cucurbitacins E and I in rats. We also describe for the first time the development of a population pharmacokinetic model of cucurbitacin E in rats.

2. MATERIAL AND METHODS

2.1. Development and validation of a sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma

2.1.1 Chemicals and reagents

The cucurbitacin E ($\geq$ 95%) and cucurbitacin I ($\geq$ 95%) standards, clobazam (internal standard) (Figure 2), and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). All solvents used were of HPLC grade. Methanol, acetonitrile, sodium chloride, monobasic sodium phosphate, and dibasic sodium phosphate were purchased from Merck (Darmstadt, Germany). Isopropanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used in the experiments was purified with the Synergy® UV water purification system (Millipore, Belford, MA, USA).

2.1.2 Chromatographic analysis

Cucurbitacin E and cucurbitacin I were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using the Acquity UPLC H-Class System®, which consists of a quaternary pump, column oven and automatic injector, coupled to a Xevo® TQ-S triple quadrupole detector (Waters, Milford, MA, USA). Cucurbitacin E, cucurbitacin I and clobazam (internal standard) were separated on a Chromolit® RP-18 column (100 x 4.6 mm, 4-µm particle size) maintained at 24ºC. The mobile phase consisted of a mixture of acetonitrile:water:methanol (32:35:33; v/v/v) eluted at a flow rate of 0.8 mL/min.

The tandem mass spectrometer with electrospray ionization operated under the following conditions: nitrogen as nebulizer gas at a flow rate of 150 L/h and argon as collision gas at a flow rate of 0.18 mL/min, capillary voltage of 2.5 kV, temperature of the ionization source of 150ºC, and desolvation temperature of 600ºC. The analyses were performed by multiple reactions monitoring (MRM) in the negative mode for cucurbitacin E (555.2>537.3 *m/z*) and cucurbitacin I (513.4>495.3 *m/z*) and in the positive mode for clobazam (301.0>256.0 *m/z*). The MassLynx 4.1 program (Waters, Milford, MA, USA) was used for data acquisition and samples quantification.

2.1.3 Preparation of the standards and quality controls

Stock solutions of cucurbitacin E and cucurbitacin I were prepared at a concentration of 100 µg/mL methanol and diluted to obtain working solutions of 2, 4, 8, 20, 40, 80, 160 and 200 ng cucurbitacin E/mL methanol and of 0.8, 1.6, 4, 10, 20, 40,

134   100, 160, 320 and 400 ng cucurbitacin I/mL methanol. The clobazam solution (internal

135   standard) was prepared at a concentration of 1000 μg/mL methanol and diluted to 500

136   ng/mL methanol.

137   The calibration curves were constructed using 50-μL blank rat plasma spiked

138   with 25 μL of each standard solution of cucurbitacin E and cucurbitacin I, resulting in

139   concentrations of 1, 2, 4, 10, 20, 40, 80 and 100 ng cucurbitacin E/mL plasma and of

140   0.4, 0.8, 2, 5, 10, 20, 50, 80, 160 and 200 ng cucurbitacin I/mL plasma.

141   The quality controls (QC) were prepared using aliquots of blank plasm spiked

142   with the standard solutions of cucurbitacin E and cucurbitacin I. The following QC

143   samples were prepared: lower limit of quantification (LLOQ; 1.0 ng/mL for

144   cucurbitacin E and 0.4 ng/mL for cucurbitacin I), low concentration (LQC; 2.0 ng/mL

145   for cucurbitacin E and 0.8 ng/mL for cucurbitacin I), medium concentration (MQC; 40

146   ng/mL for cucurbitacin E and 80 ng/mL for cucurbitacin I), and high concentration

147   (HQC; 80.0 ng/mL for cucurbitacin E and 160 ng/mL for cucurbitacin I).

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149   **2.1.4 Sample preparation**

150   Aliquots (50 μL) of rat plasma were added to 100 μL acetonitrile for protein

151   precipitation. The tubes were shaken for 30 s in a shaker and centrifuged at 6,000 rpm

152   for 2 min at 5°C. Next, 100 μL aliquots of the supernatants were transferred to vials of

153   the automatic injector of the HPLC system and 100 μL of a mixture of

154   acetonitrile:water (1:1, v/v) and 25 μL of the internal standard solution were added. The

155   vials were shaken in a shaker for 30 s and 50 μL aliquots were injected into the

156   chromatographic system for analysis.

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158   **2.1.5 Validation of the analytical method**

159   The analytical method for cucurbitacin E and cucurbitacin I in rat plasma was

160   validated according to the guidelines of the European Medicines Agency (EMEA) [22]

161   for bioanalytical methods. The following parameters were evaluated: lower limit of

162   quantitation, selectivity, carry-over, matrix effect, linearity, precision, accuracy and

163   stability.

164   The method was validated using aliquots of blank plasma derived from blood

165   samples of male Wistar rats not submitted to the experimental protocol as biological

166   matrix (blank sample). The blood samples were collected using heparin as

167   anticoagulant.

168   Calibration curves (n= 6) were obtained by spiking aliquots of 50 µL blank

169 plasma samples with cucurbitacin E and I standard solutions. The calibration curve was

170 constructed by plotting the cucurbitacin E and I/IS peak area versus cucurbitacin E and I

171 concentrations. The correlation coefficient (r) and linear regression equation were

172 calculated using the linear regression method ($1/x^2$). The lower limit of quantitation

173 (LLOQ) was defined as the lowest concentration of cucurbitacin E and I in plasma

174 quantified with acceptable accuracy and precision (coefficient of variation and percent

175 inaccuracy of less than 20%).

176   Carry-over was assessed by directly injecting an extracted blank after both

177 replicates of the upper limit of quantification of cucurbitacin E and I.

178   The matrix effect was assessed by direct comparison of the peak areas of

179 cucurbitacin E and I and the internal standard injected directly into the mobile phase,

180 and spiked into extracts originating from six diferente sources of rat plasma. The IS

181 normalized matrix factor was calculated for each matrix lot by dividing the ratio of the

182 analyte/IS response in the presence of matrix by the ratio of the analyte/IS response in

183 the absence of matrix. The coefficient of variation for the IS normalized matrix factor

184 should be less than 15%.

185   Precision and accuracy were evaluated by intra and inter-assay studies. Six

186 replicates of quality control samples were evaluated in a single analytical run (intra-

187 assay) and in three different runs on different days (inter-assay). Precision is reported as

188 the coefficient of variation (CV), that must be equal to or less than 15%, except for

189 LLOQ, for which it should be up to 20%. Accuracy is determined by percent

190 inaccuracy, excluding values higher than 15% of the nominal value, except for LLOQ,

191 for which values higher than 20% of the nominal concentration should be excluded.

192   The stability of the cucurbitacin E and I was guaranteed by two freeze (-70 $^{\circ}$C)

193 and thaw (25 $^{\circ}$C) cycles lasting 12 h each and by the evaluation of short-term stability (4

194 h at room temperature, 25 $^{\circ}$C) and postprocessing stability (12 $^{\circ}$C for 12 h). For this

195 purpose, blank plasma samples spiked with cucurbitacin E and I concentrations of LQC

196 and HQC ng/mL plasma were analyzed in six replicates. The results of the stability tests

197 are reported as accuracy in relation to freshly prepared samples. The samples were

198 considered stable when the relative error (RER, %) from the nominal concentration was

199 within ±15% and when the CV was below 15%.

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2.2. Population pharmacokinetics of cucurbitacin E in rat plasma

2.2.1. Experimental protocol

Male Wistar rats weighing 200-300 g, obtained from the Animal House of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, were used. The procedures were approved by the Ethics Committee on Animal Use of the University of São Paulo (Protocol CEUA USP 12.1.1382.53.9).

The cucurbitacin E solution administered to the animals was prepared at a concentration of 0.4 mg/mL in a mixture (5:95, v/v) of DMSO and phosphate-buffer saline (67 mM, pH 7.4). The animals (n = 6 per sampling time) were treated with a single intravenous dose (1 mg/kg) of cucurbitacin E. Serial blood samples of 200 µL (3-4 samples per animal) were collected of the tail vein of each animal at time zero, 5, 15, 30 and 45 min and 1, 2, 3, 4, 6, 8, 12, 16 and 24 h after the cucurbitacin administration. In this study were used 36 animals. The blood samples were transferred to tubes containing heparin and immediately centrifuged (2 min, 6000 rpm, 5°C) for the separation of plasma. The plasma aliquots were immediately analyzed using the method described in item 2.1.4.

2.2.2. Development of the population pharmacokinetic model

For evaluation of population pharmacokinetics, a nonlinear model of mixed effects was developed with the NONMEM v.7.3 program (ICON Development Solutions, Ellicott City, MD, USA) in the first-order conditional estimation mode with the interaction option (FOCE-I) [23] using a GNU Fortran 4.6 compiler (Free Software Foundation, Inc.) and PsN interface, version 4.4.0 (Perl-speaks-NONMEM, University of Uppsala, Sweden) [24].

Model building criteria included successful minimization without termination of the covariance step, standard error of estimates and absence of correlation between parameter estimates. Comparison of hierarchical models was based on the objective function (OF) value. A parameter was considered statistically relevant and included in the model if it decreased the OF more than 3.84 (p=0.05), following the assumption that the change in the OF after the addition of a parameter approximate a χ^2 distribution with one degree of freedom. Goodness of fit was assessed by graphical methods, including population and individual predicted vs. observed concentrations, conditional weighted residuals vs. population predicted concentrations and time.

235   The samples below the limit of quantification (BQL) were retained for model

236   building purposes. These samples were analyzed by modelling the probability that they

237   are actually below the limit of quantification [25].

238   The model was evaluated for its predictive performance using graphical criteria,

239   as assessed by visual predictive check based on 1,000 individual simulations of plasma

240   concentrations vs. time. The confidence intervals around the median 5% and 95%

241   intervals of the simulated concentrations were plotted together with the observed data to

242   visually evaluate the fit of the model to the data, its precision, and predictive

243   performance.

244

3. RESULTS AND DISCUSSION

3.1. Development and validation of the sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma

248   The present study describes for the first time the development and validation of a

249   method using LC-MS/MS for the analysis of cucurbitacin E in rat plasma and the first

250   sequential analytical method for cucurbitacin and its metabolite using LC-MS/MS.

251   The analytical method for cucurbitacin E and cucurbitacin I in rat plasma

252   permitted the sequential analysis of both cucurbitacins, with a chromatographic run time

253   of 6 min. The lowers limits of quantification (LLOQ) were 0.4 ng cucurbitacin I and 1.0

254   ng cucurbitacin E/mL plasma using plasma aliquots of only 50 µL (Figure 3). It should

255   be noted that the limit of quantification obtained for cucurbitacin I was 25 times lower

256   than that reported in the literature (10 ng/mL plasma) by Molavi et al., 2006 [21].

257   Tables 1 and 2 show the results of linearity, LLOQ, precision and accuracy

258   obtained in the validation of the sequential analytical method for cucurbitacin E and

259   cucurbitacin I, respectively, in rat plasma. The validated method showed no matrix

260   effect, considering that the coefficients of variation obtained for all matriz factor (MF)

261   values were less than 15%. Analysis of different blank plasma samples revealed the

262   absence of interference of endogenous compounds with cucurbitacin E, cucurbitacin I

263   and internal standard, indicating adequate selectivity. The two blank plasma samples

264   analyzed immediately after injection of the upper limit of quantification (ULOQ)

265   sample exhibited no residual effect. Coefficients of variation and standard errors of less

266   than 15% were obtained in the precision (CV=9.6%) and accuracy (RSE=10.3%)

267   studies (Table 1 and Table 2), indicating that the method is accurate and precise.

268   Stability tests showed that cucurbitacin E and cucurbitacin I are stable for 30

269   days when prepared in methanol (stability testing in solution). With respect to the

270   stability of cucurbitacin E and cucurbitacin I in rat plasma, the samples were stable after

271   two freeze-thaw cycles, at room temperature for up to 4 h, after processing for 12 h at

272   12ºC, and during storage at -70ºC for a period of 30 days.

273   The validated sequential analytical method for cucurbitacin E and cucurbitacin I

274   in rat plasma was applied to investigate the population pharmacokinetics of cucurbitacin

275   E and its metabolite cucurbitacin I in rats.

276

277   **3.2. Plasma concentrations of cucurbitacin E and its metabolite cucurbitacin I in**

278   **rat plasma samples**

279   The administration of a single intravenous dose of 1 mg/kg cucurbitacin E to rats

280   resulted in plasma concentrations of cucurbitacin E above the LLOQ up to 16 h after

281   administration. Figure 4 shows the chromatograms obtained for the analysis of a plasma

282   sample collected 3 h after intravenous administration of 1 mg/kg cucurbitacin E to a rat.

283   However, the plasma concentrations of cucurbitacin I remained below the LLOQ (0.4

284   ng/mL plasma) in all samples collected up to 24 h after the administration of

285   cucurbitacin E.

286   Studies on the metabolism of cucurbitacin E in human liver microsomes and in

287   human plasma have shown the formation of cucurbitacin I [17,16]. Saade et al. (2009)

288   [16] suggested the participation of paraoxonase in the hydrolysis of cucurbitacin E to

289   cucurbitacin I in human plasma. However, the concentrations of esterases in human

290   plasma differ from those found in rat plasma; for example, the concentration of

291   paraoxonase is 2-fold higher in human plasma than in rat plasma [26]. Thus, it is

292   possible that rat plasma is unable to hydrolyze cucurbitacin E to cucurbitacin I, a fact

293   that would explain the plasma concentrations of cucurbitacin I below the LLOQ in the

294   study of intravenous administration of cucurbitacin E to rats.

295

296   **3.3. Development and validation of the population pharmacokinetic model of**

297   **cucurbitacin E in rat plasma after intravenous administration**

298   This is the first study describing a population pharmacokinetic model for the

299   class of cucurbitacins, particularly cucurbitacin E administered intravenously. The

300   plasma concentration-time profile of cucurbitacin E after a single intravenous dose

301   administration to rats (Figure 5) and the release time (D) of 0.45 h estimated with the

302 population pharmacokinetic model (Table 3) show that the pharmacokinetic behavior of

303 cucurbitacin E resembles more a controlled release or infusion profile rather than the

304 bolus intravenous administration used in the experimental study. Based on this

305 observation, the population pharmacokinetic model of cucurbitacin E was developed

306 assuming administration as an intravenous infusion. An overview of the goodness-of-fit

307 is shown in the Figure 6. The plots show that individually predicted concentrations are

308 unbiased and that residual errors are randomly distributed around mean zero.

309 Cucurbitacin E is not soluble in polar solvents and therefore requires prior

310 solubilization in DMSO followed by dilution in phosphate-buffered saline. When

311 diluted in aqueous buffer, analytes soluble in DMSO form a homogenous solution, a

312 homogenous suspension, or a heterogenous suspension with the observation of

313 precipitation [27]. The behavior of cucurbitacin E in the phosphate buffer-DMSO

314 mixture (95:5, v/v) at the concentration used in the pharmacokinetic study (0.4 mg

315 cucurbitacin E/mL) resembles a homogenous suspension, a fact explaining the release

316 time of 0.45 h after intravenous administration. Concentrations higher than 0.4 mg

317 cucurbitacin E/mL were not used because of the change from a homogenous suspension

318 to a heterogenous suspension.

319 An overview of the goodness-of-fit is shown in the Figure 6. The plots show that

320 individually predicted concentrations are unbiased and that residual errors are randomly

321 distributed around mean zero. The high conditional weighted residuals shown are a

322 consequence the high inter-individual and experimental variability of the data in 6

323 subjects. This variability led the predicted concentrations to deviate considerably from

324 the observed concentrations. However, these deviations have not affected the estimation

325 of the pharmacokinetic parameters. In fact, the model parameter estimates proved to be

326 robust. During the analysis, the exclusion of the outliers resulted in estimates values

327 similar to the ones obtained with the full data set.

328 Figure 7 illustrates the visual predictive check of the population pharmacokinetic

329 model of cucurbitacin E in rat plasma after intravenous administration. Additionally, the

330 figure indicates the percentage of BQL samples for each sample time, in case they are

331 present, to highlight their influence on the prediction of the drug concentrations. The

332 pharmacokinetic profile of the median plasma concentrations of cucurbitacin E was

333 described by a two-compartment model with first-order distribution and elimination.

334 The pharmacokinetic parameter estimates, interindividual variability, residual

335 variability of the model and relative standard deviation (RSD%) of these estimates are

shown in Table 3 for release time (D), volume of distribution from the central compartment (Vc) and from the peripheral compartment (Vp), clearance (Cl), and intercompartmental clearance (Q).

The population pharmacokinetic profile of cucurbitacin E administered intravenously to rats (Table 3) suggests the need to develop formulations that result in a solubility compatible with the requirements of preclinical pharmacokinetic studies. Additionally, studies on the metabolism of cucurbitacin E and *in vitro* membrane transporters using human cells are necessary.

4. CONCLUSION

The sequential analytical method for cucurbitacin E and cucurbitacin I in rat plasma using LC-MS/MS is precise, accurate and sensitive, permitting its application to preclinical pharmacokinetic studies of single intravenous dose. The cucurbitacin I plasma concentrations remained below the LLOQ in all samples collected after intravenous administration of cucurbitacin E. It is possible that rat plasma is unable to hydrolyze cucurbitacin E to cucurbitacin I because of differences in esterase concentrations between human and rat plasma.

The pharmacokinetic profile of cucurbitacin E administered intravenously to rats was described by a two-compartment model with first-order distribution and elimination and the following pharmacokinetic parameters were obtained: volume of distribution of 27.22 L, clearance of 4.13 L/h, and elimination half-life of 4.57 h. In addition, the pharmacokinetic behavior of cucurbitacin E administered intravenously to rats resembled an infusion, with a release time of 0.45 h due to the low solubility of cucurbitacin E prepared in a mixture of phosphate-buffered saline (pH 7.4) and DMSO (95:5, v/v).

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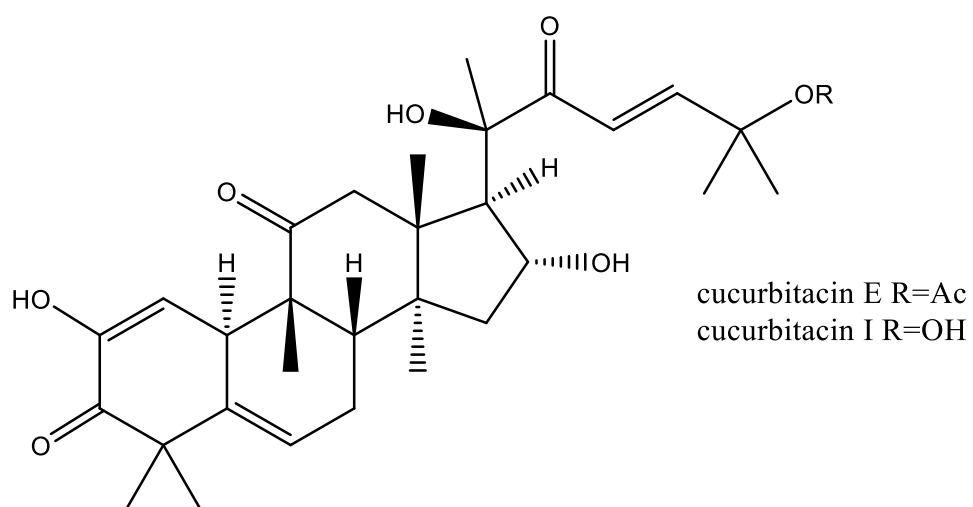
cucurbitacin E R=Ac
cucurbitacin I R=OH

Figure 1. Chemical structure of cucurbitacin E and cucurbitacin I.

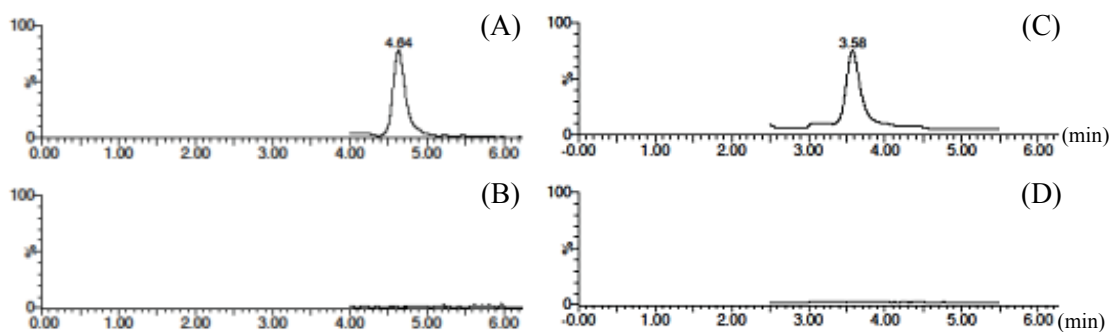**Figure 2.** Chemical structure of clobazam (internal standard).

Figure 3. Representative MRM chromatograms for cucurbitacin E and cucurbitacin I. (A) Chromatogram of rat plasma spiked with 1.0 ng/mL (LLOQ) of cucurbitacin E; (B) Chromatogram of blank plasma monitored in the transition of cucurbitacin E (555.2>537.3); (C) Chromatogram of rat plasma spiked with 0.4 ng/mL (LLOQ) of cucurbitacin I; (D) Chromatogram of blank plasma monitored in the transition of cucurbitacin I (513.4>495.3).
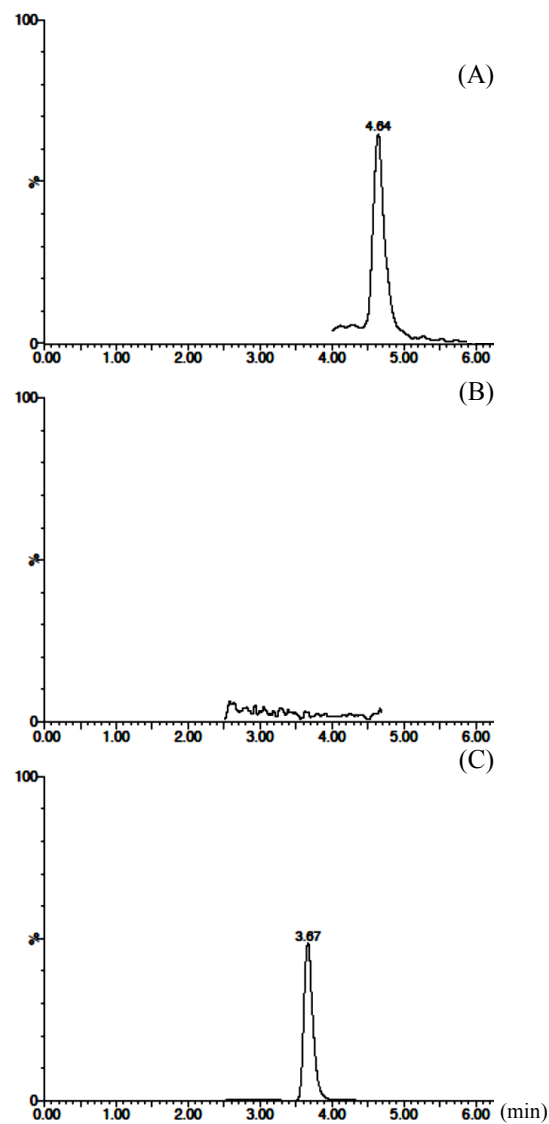
Figure 4. Chromatograms obtained for the analysis of a plasma sample collected 3 h after intravenous administration of cucurbitacin E (1 mg/kg) to a Wistar rat. (A) Chromatogram showing the transition of cucurbitacin E (555.2>537.3). (B) Chromatogram showing the transition of cucurbitacin I (513.4>495.3). (C) Chromatogram showing the transition of clobazam (internal standard) (301.0>224.0).

Figure 5. The plasma concentration-time profile of cucurbitacin E after a single intravenous dose administration to rats.
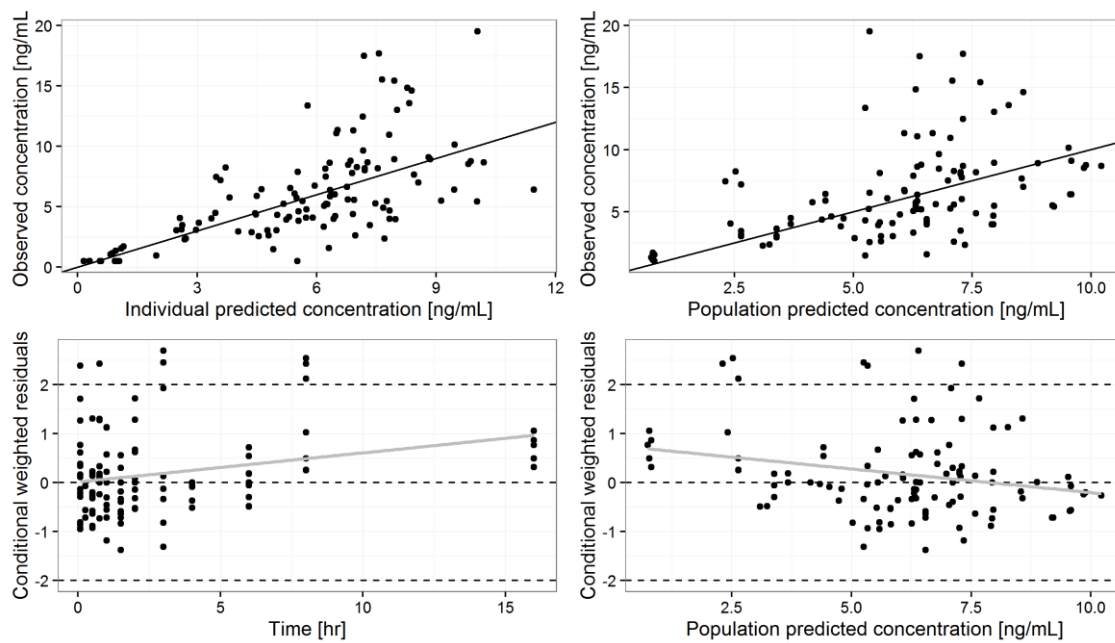
Figure 6. Goodness-of-fit. The plots show that individually predicted concentrations are unbiased and that residual errors are randomly distributed around mean zero.
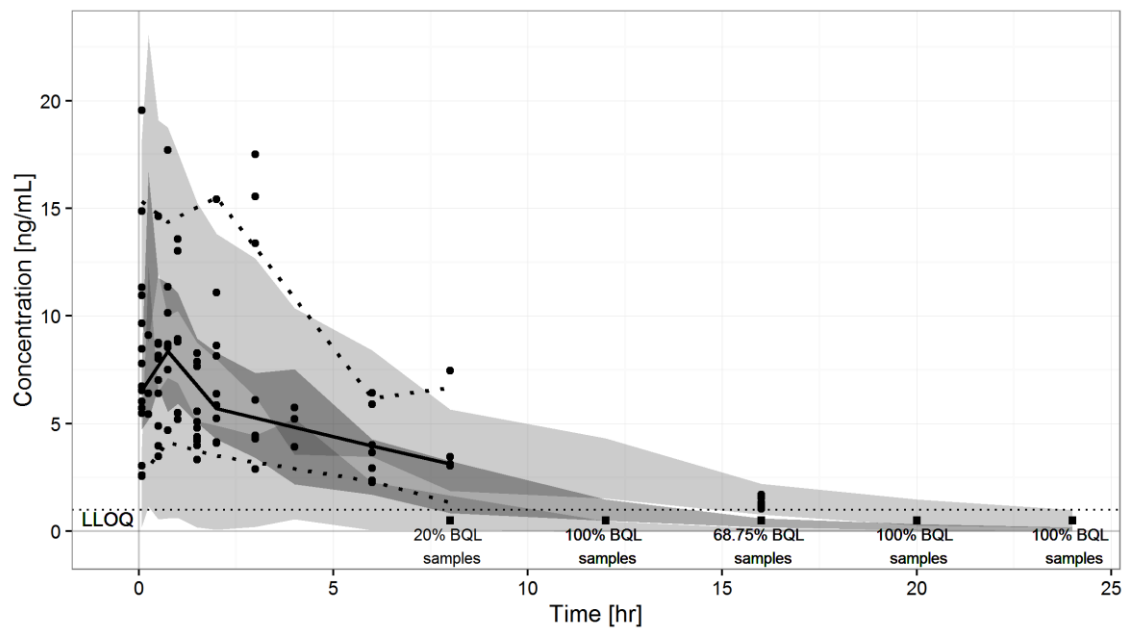
Figure 7. Visual predictive check. The observed plasma concentrations are shown as dots. The solid line represents the median of the observed plasma concentrations. The dotted lines indicate the 5th and 95th percentiles of the observed plasma concentrations. The dark grey shaded area represents the confidence interval around the median of the predicted plasma concentrations. The light grey shaded areas show the confidence interval around the 5th and 95th percentiles of the predicted plasma concentrations.

Table 1. Validation parameters of the analytical method for cucurbitacin E in rat plasma.

Parameter			
Linearity (ng/mL)	1.0 - 100.0		
Straight-line equation	y=0.0277031x+0.0352023		
Linear correlation coefficient	r=0.991606		
(ng/mL)	**Measured concentration (ng/mL) ± S.D**	**Between-run precision (n=18)** (CV %)	**Within-run precision (n=6)** (CV %)
1.0	1.12 ± 0.13	12.78	7.26
2.0	2.17 ± 0.20	9.59	10.06
40.0	39.10 ± 1.11	8.71	12.2
80.0	82.34 ± 3.24	13.85	13.43
	Measured concentration (ng/mL) ± S.D	**Between-run accuracy (n=18)** (RSE %)	**Within-run accuracy (n=6)** (RSE %)
1.0	1.01 ± 0.07	10.66	1.6
2.0	2.04 ± 0.05	7.87	3.0
40.0	40.24 ± 0.68	4.72	4.47
80.0	79.32 ± 1.56	-5.8	2.1

CV: coefficient of variation [(standard deviation/mean) x 100]; RSE: relative standard error [(mean - nominal value)/nominal value x 100]; r: linear correlation coefficient.

Table 2. Validation parameters of the analytical method for cucurbitacin I in rat plasma.

Parameter			
Linearity (ng/mL)	0.4 - 200.0		
Straight-line equation	y=0.00202823x+0.00490914		
Linear correlation coefficient	r=0.993945		
	Measured concentration (ng/mL) ± S.D	**Between-run precision (n=18)** (CV %)	**Within-run precision (n=6)** (CV %)
(ng/mL)			
0.4	0.48 ± 0.12	9.32	12.45
0.8	0.76 ± 0.22	11.2	9.15
80.0	83.12 ± 3.18	7.9	11.3
160.0	164.73 ± 6.43	10.34	8.64
	Measured concentration (ng/mL) ± S.D	**Between-run accuracy (n=18)** (RSE %)	**Within-run accuracy (n=6)** (RSE %)
0.4	0.35 ± 0.09	8.65	8.45
0.8	0.87 ± 0.56	7.6	9.5
80.0	81.12 ± 4.03	10.15	6.8
160.0	162.17 ± 9.56	11.25	-5.4

CV: coefficient of variation [(standard deviation/mean) x 100]; RSE: relative standard error

[(mean - nominal value)/nominal value x 100]; r: linear correlation coefficient.

Table 3. Estimates of the population pharmacokinetic model of cucurbitacin E (1 mg/kg) administered intravenously to rats.

	Typical value (θ)	RSD%	IIV (CV%)	RSD%
Clearance (L/h)	4.13	5.1%	28.8%	6.6%
Vc (L)	0.815	14.2%	--	--
Q (L/h)	110	9.7%	48.7%	9.8%
Vp (L)	26.4	6.2%	--	--
D (h)	0.452	8.6%	--	--
σ^2	0.562	9.2%	--	--

Vc: central volume of distribution; Q: intercompartmental clearance; Vp: peripheral volume of distribution; D: release time; IIV: interindividual variability (expressed as the coefficient of variation: $CV(\%) = \sqrt{\omega^2} \cdot 100$); χ^2: variance of arwhere $Y = F + F \cdot \varepsilon$); RSD%: relative standard deviation.